IN VITRO CANCER METHODOLOGY FOR HUMAN CELL LINES

1. Cells are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2mM L-glutamine.
2. Dependent upon cell doubling time, between 5,000 and 40,000 cells are inoculated into 96 well microtiter plates in a volume of 100 uL per well.
3. The plates are incubated at 37^{o}C, 5 % CO2, 95 % air, and 100 % relative humidity for 24 hours prior to the addition of the experimental drug.
4. After the 24 hour incubation, two (2) plates of each cell line are fixed *in situ* with TCA to establish the cell population at time of drug addition (Tz).
5. Prior to use, the experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and frozen.
6. At time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 ug/ml gentamicin. An additional four (4) 10-fold or ½ log serial dilutions are made for a total of five (5) drug concentrations plus a control.
7. An aliquot of 100 ul of each drug dilution is added to the appropriate well that already contains 100 ul of medium containing the cells.
8. The plates are then incubated for 48 hours at 37^{o}C, 5 % CO2, 95 % air and 100 % relative humidity.
9. FOR ADHERENT CELLS:
   Cells are fixed *in situ* by the gentle addition of 50 ul cold (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4^{o}C. The supernatant is discarded, plates washed five (5) times with tap water and air dried. Then Sulforhodamine B (SRB) solution (100 ul) at a 0.4 % (w.v) in 1 % acetic acid is added to each well, an the plates incubated for 10 minutes at room temperature. Unbound dye is removed by washing five (5) times with 1 % acetic acid and the plates are air dried. The bound stain is solubilized with 10 mM trizma baseanad the absorbance is read on an automated plate reader at a wavelength of 515 nm.

   FOR SUSPENDED CELLS:
   Methodology is the same as above except that the assay is terminated by the fixing of the settled cells at the bottom of the wells by the gentle addition of 50 ul of 80 % TCA (final concentration, 16 % TCA).

10. Percent growth inhibition is calculated using the seven absorbance measurements [time zero, (Tz), growth control, (C), plus the test growth at the five drug concentration levels (Ti)] as follows:

    [(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti>/=Tz
    [(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz

    Three (3) dose response parameters are calculated for each test agent. Growth inhibition of 50 % (GI50) is calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, which is the concentration of drug giving a 50 % reduction in the net protein increase (as measured by SRB staining) in the control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from Ti = Tz. The LC50 (concentration of test agent resulting in a 50 % reduction in the measured protein at the end of the drug treatment as compared to tht at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = -50. For each of these parameters values are calculated if the level of activity is reached; however, if the effect is not reached or is exceeded , the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

IN VITRO CANCER EXPERIMENTAL PROCEDURES (GENERAL)

Cell Line Inoculation

All 60 human tumor cell lines, available are maintained in RPMI-L 640 media supplemented with 5% fetal bovine serum and 1 % L-glutamine and used up to the 20^{th} passage, when a new cryopreserved vial of cells started. Cells are grown in incubators with 100% humidity and 5% $C0_2$. Cell lines are split 3-5 days prior to inoculation to produce a flask of cells that is in log growth and approximately 70% confluent on the day of inoculation.

1. Calculate the number plates to inoculate per cell line and the number of ml of cell suspension required:

    a. (Number of vertical drugs x number of horizontal drugs x 2) = total # of plates

    b. (total number of plates x 7.Oml) = ml of cell suspension required

2. Remove the flasks to be used from the incubator and examine under the microscope for contamination (bacterial, fungal), confluency (approximately 70-80%), general cell health (do the cells look normal, amount of floating cells).

3. General trypsinization procedure (varies depending on the cell line, leukemia cell lines grow in suspension and require no trypsinization):

    a. Aspirate the media from the flask.

    b. Add approximately 2ml of trypsin to the flask and rinse the cell monolayer.

    c. Aspirate the trypsin from the flask.

    d. Add approximately 2ml of trypsin to the flask to cover the cell monolayer.

    e. Depending on the cell line, allow the trypsin to remain on the cell monolayer at room temperature or in the incubator for approximately 2-5 minutes.

    f. After exposure to the trypsin, gently tap the flask to dislodge the cells from the flask.

    g. Add approximately 20ml of media to the flask and gently pipet the cell suspension to break up any clumps of cells and to rinse the side of the flask to remove all of the cells.

    h. Transfer the cells to a 50ml conical tube.

4. Place the 50ml conical tube containing the cell suspension into a centrifuge and spin the cells for 5 minutes at 1000rpm to pellet the cells at the bottom of the tube.

5. Aspirate the media from the conical tube being sure to leave the cell pellet intact.

6. Resuspend the cell pellet in approximately 20-30ml of media and gently pipet the solution to evenly suspend the cells in the media and to break up any remaining cell clumps.

7. Count the number of cells/ml in the cell suspension using a Coulter Counter by:

    a. Place 0.5ml cell suspension from conical tube into 19.5ml of Isoton 11 solution in a Coulter sample cup.

    b. Make 2-4 counts on the Coulter Counter of the cell suspension in the sample cup.

    c. Calculate the average count from the 2-4 counts.

    d. Multiply the average count by the dilution factor of the cells in the lsoton 11 solution (40 for a 1:40 dilution).

    e. Multiply the number generated in step d by 2 if a 500ml sample was measured or by 10 if a 100ml sample was measured, the result is the number of cells/ml of the suspension in the conical tube.

8. Calculate the number of ml of cell suspension from conical tube to be placed in media to obtain the correct cell density by:

    $$\frac{\text{(Inoculation Density x 10 x Number of ml of cell suspension required)}}{\text{total number of cells in suspension}} = \text{ml of cell suspension}$$

9. Calculate the number of ml of media to add to the result of step 8:

    (Total amount of cell suspension required - ml of cell suspension from step 8) = ml of media

10. Add the amount of cell suspension calculated in step 8 to the amount of media calculated in step 9 and mix thoroughly to give a homogenous cell suspension for inoculation.

11. Inoculate the cells onto the plates as shown in figure 1 (cell inoculation plate format), by putting 100ml of cell suspension into the appropriate wells.

Drug Addition

There are three primary drug dilution schemes that are used for the vertical and horizontal drugs, log dilutions (1:10), half-log dilutions (1:3.16) and 1:2 dilutions. The drugs are either sent as clinical formulations or are prepared in a drug preparation laboratory where they are usually diluted in 100% DMSO at a concentration of 400X the high test concentration on the plate.

1. Media containing antibiotic is used for mixing and diluting of drugs to reduce the amount of bacterial contamination. To prepare the media add 530ml of gentamycin to a bottle of complete media (1 ml/ml) and swirl the contents of the bottle to mix.

2. Determine the minimum amount of media needed to add the high concentration to all of the plates for a particular drug:

    a. if adding l00ml of drug/well for vertical drugs:

        # of ml of media in high test [ ] = (# of plates x 1.6ml)

    b. if adding 50ml of drug/well for vertical drugs:

        # of ml of media in high test [ ] = (# of plates x 0.8mi)

    c. if adding 100ml of drug/well for horizontal drugs: # of ml of media in high test [I = (# of plates x 2.2ml)

    d. if adding 50ml of drug/well for horizontal drugs: # of ml of media in high test [ ] = (# of plates x 1.1 ml)

3. From the result in step 2 add 3-5ml as a safety factor since the result in step 2 is the minimum number of milliliters needed. This result gives you the number of ml of media to place in all of the tubes in your series except for the highest dose tube.

4. To determine the amount of media to place in the high concentration dose tube:

    a. for a 1:10 divide the result in step 3 by 9 to give you the extra number of milliliters of media to add to the result in step 3.

    b. for a 1:3.16 divide the result in step 3 by 2.16 to give you the extra number of milliliters of media to add to the result in step 3.

    c. for a 1:2 multiply the result in step 3 by 2 to give you the extra number of milliliters of media to the result in step 3.

5. Fill up the series of tubes with media, the tube containing the high concentration of drug gets the amount of media calculated in step 4, the other tubes in the series gets the amount of media calculated in step 3.

6. If the drug is frozen remove the drug from the freezer and rapidly thaw by placing in a water bath or incubator, or if the drug is powder solubilize the drug in the proper solvent.

7. After the drug has been thawed or prepared, do the proper dilution of drug into the high concentration tube. To determine the amount of drug to be placed into the tube:

    ml of drug = (amount of media in tube) + (dilution factor)

8. Add the appropriate amount of drug to the high concentration tube and thoroughly mix the

solution in the tube.

9. To determine the amount of drug solution to transfer from tube to tube throughout the serial dilution:

    a. for a 1:10 dilution divide the amount of media in the high concentration tube by 10.

    b. for a 1:3.16 dilution divide the amount of media in the high concentration tube by 3.16.

    c. for a 1:2 dilution divide the amount of media in the high concentration tube by 2.

10. Take the amount of drug solution from the high concentration tube (as calculated in step 9) and place it into the next highest concentration tube, thoroughly mix the contents.

11. Continue throughout the dilution series transferring the same amount of drug from one tube to the next.

12. Add the drug to the plate(s) following the correct drug addition plate format for either the vertical drug or the horizontal drug.

13. If drugs are to be removed from the plate after a specific period of time:

    a. Place an 8 channel manifold into a 70% ETOH bath.

    b. Remove the plates that have drug to be removed from the incubator.

    c. Remove the 8 channel manifold from the alcohol bath and place onto the end of a vacuum tube hooked to a waste bottle.

    d. Allow the alcohol in/on the manifold to either be aspirated into the waste jar or evaporate.

    e. Carefully aspirate the media/drug from the plates by slowly inserting the manifold into the wells going as close to the bottom of the plate without touching the cell monolayer.

    f. When the media/drug has been removed, re-add media or drug to the plates and return the plates to the incubator.

Plate Fixation

At the end of the assay cellular protein is 'fixed' to the bottom of the plate with 50% Trichloroacetic acid (TCA) for adherent cell lines or 80% TCA for suspension cell lines. To fix the cellular protein to the plate:

1. To make 50% TCA stock solutions, add 500 grams of TCA crystals to 1.0 liters of deionized water. To make 80% TCA stock solutions, add 500 grams of TCA to 0.625 liters of deionized water. Place into a bottle and store in the refrigerator until ready to use. TCA must be cold

when used.

2. Remove the plates to be fixed from the incubator.

    a. For adherent cell lines, add 50ml of cold 50% TCA to each well of the plate, do not add the TCA too quickly as shearing of cells from the plate bottom could occur.

    b. For suspension cell lines, add 50ml of cold 80% TCA to each well of the plate by dripping the TCA into the well.

3. Place the plates in the refrigerator for a minimum of 1 hour and a maximum of 3 hours.

4. After 1 hour of refrigeration, remove the plates from the refrigerator and remove the liquid from the wells by inverting the plates over a plastic pan.

5. Rinse the plates 5 times with water, blotting plates on paper towels after rinsing.

6. Lay plates on trays, face up, and allow to dry. The drying process, at room temperature takes approximately 12-24 hours.

Plate Staining

The fixed cellular protein that is attached to the plate is stained with sulforhodamine B (SRB).

1. Make a solution of 1 % glacial acetic acid (1 part glacial acetic acid into 99 parts water) in an amount that you will need to stain your set of plates.

2. Make a 0.4% solution of SRB by dissolving 0.004 grams of SRB into 1 ml of 1 % acetic acid and mix thoroughly.

3. Add 100ml of the SRB solution to each well of the plates to be stained.

4. Leave the stained plates at room temperature for a minimum of 5 minutes to allow for complete staining of cellular protein.

5. Rinse the excess stain from the plates by rinsing 3 times with a 1 % glacial acetic acid solution.

6. Blot the rinsed plates on paper towels to remove any remaining acetic acid.

7. Lay the plates on trays, face up and allow to air dry for approximately 12-24 hours.

Plate Reading

The bound SRB stain is solubolized with 10mM Trizma base and optical densities are recorded.

1. Prepare a solution of I00mM Trizma base (Tris) by dissolving 1.21 grams of tris into 1.0 liter of water.

2. Add 100ml of the 10mM tris to each well of the plates to be read.

3. Place the plates onto a plate shaker and shake the plates until all of the bound SRB is into solution.

4. Read and record the plate optical densities on a microtiter plate reader at a wavelength of 515nm.

DATA ANALYSIS

The optical densities that are recorded are stored in an ASCII text file along with experiment identification and plate number for each plate. These data are then imported into an EXCEL spreadsheet where percent treated/control values are calculated along with a measure of synergy/antagonism.

1. The ASCII file is opened in EXCEL along with the synergy.xls spreadsheet.

2. The plate and drug information is entered into the spreadsheet in a form shown in figure 4.

3. The raw optical densities are then copied from the ASCII data and pasted into the appropriate cells of the spreadsheet. For plate 1, optical densities are pasted into the cell range of E2:P9 and for plate 2, optical densities are pasted into cell range El 1:Pl 8.

4. Average optical densities minus backgrounds are calculated for the control wells and the test wells for each concentration of horizontal and vertical drugs by using the equation:

$$\text{average OD - bk} = \left(\frac{\Sigma \text{ OD's for well group}}{\text{number of wells in group}}\right) - \left(\frac{\Sigma \text{ OD's for background group}}{\text{number of wells in background group}}\right)$$

5. Percent Treated/Control (%T/C) values are calculated for the vertical drug alone (one dose response curve made up of 8 data points for the vertical drug), for the 5 concentrations of the horizontal drug tested in combination with the vertical drug (5 dose response curves made up of 8 data points for the vertical drug) and for the horizontal drug alone (one dose response curve made up of 5 data points for the vertical drug.